Overexpression of GNB2L1 is associated with poor prognosis in cervical cancer patients

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Abstract

Background: Among women, cervical cancer (CC) is the fourth most prevalent cancer worldwide. Multiple bio-makers have been identified for tumor diagnosis and prognosis. However, no single bio-maker for CC detection exists that is both highly sensitive and specific. In this report, we explored the role of guanine nucleotide-binding protein subunit beta-2-like 1 (GNB2L1) in CC progression in Uyghur women.

Methods: In the present study, 132 patients with cervical squamous cell carcinoma (CSCC), 56 cases with cervical intraepithelial neoplasia (CIN) were enrolled. GNB2L1 mRNA and protein expression levels were analyzed in CSCC, CIN, and normal cervical (NC) tissues and in CC cell lines using qRT-PCR, western blot, and immunohistochemistry. Stable GNB2L1-knockdown CC cells (shGNB2L1) were also created and lipid accumulation was measured with Oil Red O staining. Additionally, changes in GNB2L1 gene copy number were investigated with qRT-PCR and sequencing. A gene regulatory network was constructed using the STRING database. Finally, patient survival was assessed with Kaplan-Meier and log-rank tests.

Results: Our results indicated that GNB2L1 mRNA and protein are overexpressed in CSCC tumor tissues when compared to CIN and NC tissues. GNB2L1 expression was associated with the degree of cell differentiation ($P = 0.001$), lymph node metastasis ($P = 0.049$), and the clinical stage ($P = 0.017$). Oil Red O staining showed that lipid levels were lower in shGNB2L1 cells, and that GNB2L1 DNA copy number in CC tissues ($29,7200 \pm 9,815.061$) was significantly higher when compared to NC tissues ($19,5100 \pm 5,676.392$ [$T = -2.770$, $P = 0.009$]). Furthermore, patients with CSCC and high GNB2L1 expression demonstrated shorter overall survival times when compared to patients with low GNB2L1 expression ($P = 0.002$). Multivariate analysis indicated that GNB2L1 overexpression is an independent prognostic factor in patients with CSCC.

Conclusions: Overexpression of GNB2L1 may contribute to tumor progression; therefore, it is a potential prognostic biomarker for CSCC patients.

Background

Cervical cancer (CC) is the fourth most prevalent cancer affecting women, and the incidence and mortality rates vary with geographical location [1]. Indeed, large disparities in the prevention and management of CC exist throughout the world. In the year 2018, 570,000 new cases of CC and 311,000 CC-associated deaths were reported worldwide. Intriguingly, 87 % of all cases occurred in developing countries [2]. The Xinjiang region has one of the highest rates of CC incidence in China, and the CC incidence can be up to 3.4 times greater in Uyghur woman than in Han women [3–4]. Despite advances in surgery and chemotherapy over the last 20 years, the overall survival rate for patients with CC remains largely unchanged. The high mortality rate of CC is primarily attributed to late diagnoses when the disease has already progressed to an advanced state. In these cases, the 5-year survival rate is only 8–12%. In contrast, patients that are accurately diagnosed at an earlier stage or with precancerous diseases have a 5-year survival rate of 90%. Poor survival is primarily due to uncontrolled lymph node metastasis,

which means that curative surgery is not a viable option for more than 50% of patients at the time of diagnosis [5–6]. Therefore, investigating the pathogenesis of CC to identify novel biomarkers is crucial.

Guanine nucleotide-binding protein subunit beta–2-like 1 (GNB2L1), which is also known as the receptor for activated protein kinase C, is an intercellular scaffold protein with a propeller-like structure of seven WD40 repeats. The protein is highly expressed on several types of the malignant tumors and is involved in multiple intracellular signal transduction pathways [7–9]. Notably, GNB2L1 is an excellent predictor for the clinical prognosis of oral squamous cell carcinoma (OSCC) and pancreatic ductal adenocarcinoma [10–11]. Recently, GNB2L1 was also identified as a novel factor that was required for adipocyte differentiation and metabolic disorders [12].

However, the exact mechanisms of aberrant GNB2L1 expression and its role in CC remain unclear.

The aim of the current study was to examine GNB2L1 expression in CC tumor tissues, cervical intraepithelial neoplasia (CIN), and normal cervical (NC) tissues and to compare the GNB2L1 expression patterns with clinic pathological characteristics. The prognostic value of GNB2L1 as a biomarker for CC patients was then evaluated.

Methods

Tissue specimens

Fresh or formalin-fixed and paraffin-embedded cervical tissue specimens were collected from Uighur women with CSCC and CIN, or from women without cervical diseases that underwent hysterectomy surgery. All CC patients between June 2010 and March 2013 at the First Affiliated Hospital of Xinjiang Medical University and the First Hospital of Kashi were asked to participate in our study during their initial visit to the Department of Gynecology. All cancer and control patients were provided with written informed consent, and the clinical study was approved by the ethics committee of the First Affiliated Hospital of Xinjiang Medical University.

Gynecological examinations were performed in all CC patients and staging was completed in accordance with the International Federation of Gynecology and Obstetrics (FIGO) criteria. In addition, experienced pathologists reviewed slides, which resulted in the inclusion of 220 cases of formalin-fixed, paraffin-embedded (FFPE) tissue specimens. The FFPE specimens (or fresh frozen cervical tissues, described below) were collected during the initial visit to the outpatient department, at a gynecologic examination, or after operative treatment under general anesthesia. Among the patients with CSCC that were enrolled in this study, 74 were FIGO stage $\leq$ II and 58 were FIGO stage $>$ IIIA. There were 60 cases that were pathologically characterized as well-differentiated and 72 that were characterized as moderately or poorly differentiated tumors. Lymph node metastasis was documented in 55 tumor patients. A total of 56 patients with CIN were selected for this study, including 20 cases with CIN I-II and 36 with CIN-III. The median age of the CC patients was 48.5 years (IQ range 25–67 years). Control cases (n = 32) were obtained from patients without a history of cervical lesions or any other form of cancer that had a

planned hysterectomy for nonmalignant reasons. Indications for hysterectomy were fibroids, uterine prolapse, adenomyosis, or a combination of fibroids and uterine prolapse.

In addition, 40 frozen biopsies, including 20 CSCC cases and 20 normal adjacent tissues (NATs), were collected according to the criteria described above for gene transcription and GNB2L1 copy number studies.

Immunohistochemistry

To examine the staining patterns of various target proteins in CSCC, CIN, and NC tissues, fixed preparations were dewaxed in dewaxing agent (Zhong Shan Goldenbridge Biotechnology Co. Ltd, Beijing, China), rehydrated in alcohol, and then blocked with an endogenous peroxidase inhibitor (Zhong Shan Goldenbridge Biotechnology Co. Ltd, Beijing, China) at room temperature for 30 min. The samples were then incubated with primary antibodies against GNB2L1 (Abcam, Cambridge, MA, USA) overnight at 4°C. Immunohistochemistry of clinical samples was performed as previously reported [11]. Two experienced pathologists independently scored the staining patterns, which were semi-quantitatively estimated according to the staining intensity and distribution. A score of 9–12 was classified as high expression, a score of 5–8 was classified as moderate expression, and a score 1–4 was classified as low expression.

RNA extraction and quantitative real-time (qRT)-PCR

Total RNA from the clinical specimens and CC cells was extracted with TRIzol reagent (Invitrogen) and reverse transcribed into 2 mg of cDNA with the Revert Aid First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). Real-time (RT)-PCR was performed using the SYBR Green Premix PCR Master Mix (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocols. The quantitative RT-PCR (qRT-PCR) analysis was performed using the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) on an Applied Biosystems ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Gene expression levels were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference. The average relative change in gene expression was calculated using 3–5 determinations by relative quantification and applying the delta-delta cycle threshold method. All reactions were performed in triplicate. The oligonucleotide primers for human GNB2L1 and GAPDH were as follows: GNB2L1-F: 5'-TGAGTGTGGCCTTCTCCTCT-3'; GNB2L1-R: 5'-GCTTGCA GTTAGCCAG GTTC-3'; GAPDH-F: 5'-GGACCTGACCTGCCG TCTAG-3'; GAPDH-R: 5'-GTAGCCCAG GATGCCCT TGA-3'.

Genomic DNA isolation and quantitative DNA gene copy analysis

Genomic DNA was extracted from human cervical tissue samples using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) and the GNB2L1 gene was detected with qRT-PCR according to the manufacturer's protocols [13]. The oligonucleotide primers for human GNB2L1 were as follows: GNB2L1-F: 5'GCAGCAACCCTATCATCGTC3' and GNB2L1-R: 5'CGGTAGCCATTTCTC ATTCA3'. The PCR products were linked to the pEASY-T1 vector, which was ampicillin and kanamycin resistant. The identification of successful cloning was performed using primers that were specific to the target genes. The sequencing reaction was performed with the universal primers, M13-F and M13-R, and the cycle threshold value of each sample was detected with qRT-PCR to calculate the GNB2L1 copy number.

Cell culture and lentiviral infection

Two human CC cell lines (SiHa and C33a) and a human immortalized cervical squamous epithelial (H8) cell line were purchased from Shanghai Cell Collection (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (BI, Cromwell, CT, USA) and maintained at 37°C with 5% CO₂. The cells were routinely passaged at a density of 90%. The culture medium was supplemented with 10% fetal bovine serum (FBS) and 100 units/mL of penicillin and streptomycin (BI, Cromwell, CT, USA). The lentiviral vector constructs and the lentivirus were produced by GENECHM (Shanghai, China). The cell lines were treated with two RNAi sequences targeting GNB2L1, which were as follows, shGNB2L1-2: 5'-GCAAGATCATTGTAGATGA-3' and shGNB2L1-2: 5'-GCTAAAG ACCAACCACATT-3'. Nonsense shRNA was the negative control. Briefly, the cell lines were plated in 6-well plates with serum-free DMEM and incubated at 37°C in the presence of 8 µg/mL polybrene for 72 h, according to the manufacturer's guidelines. The medium was then removed and replaced with culture medium that contained 10% FBS. The cells were then continuously cultured for 6–8 days, followed by flow cytometry selection.

Antibodies and western blot analysis

Lysate protein concentrations were quantified with the BCA protein assay kit (Bio-Rad Hercules, CA, USA) and the appropriate amount of lysate was loaded into a 10% polyacrylamide gel. The samples were then resolved by SDS-PAGE and the proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated with antibodies against GNB2L1 (ab62735, Abcam, Cambridge, MA, USA) and GAPDH (Proteintech, Rosemont, IL, USA). Protein detection was performed with the Western Breeze Chromogenic Immunodetection System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

Gene regulatory network construction

To identify network pathways related to differentially expressed proteins or genes in CSCC, bioinformatics and the STRING database were used to construct an interaction network. First, genes with consistent expression and significant expression differences from our previous proteomic and microarray studies

were selected as the experimental differential genes [14]. Then, the 19 differential genes were inputted into the STRING database to identify potential relationships among the protein subunits and to construct a network of their interactions.

Oil Red O staining

Stable GNB2L1-knockdown CC cells (shGNB2L1) were washed with phosphate-buffered saline (PBS) and fixed with Paraformaldehyde Fix Solution for 20 min at 37°C. The cells were then washed with PBS, followed by a 60% isopropanol wash for 1 min. Next, the cells were stained with a filtered 0.3% Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) solution for 30 min at 37°C, followed by washing in PBS. The total cell number was obtained by counting. Twenty random fields were analyzed (×20) using a Nikon ECLIPSE TS100 epifluorescence microscope. To quantify the lipid content, the cell-incorporated Oil Red O was extracted in 6-well plates by shaking in 500 µL of isopropanol for 10 min at room temperature. The supernatant was then collected and absorbance at 490 nm was measured with a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analyses

All statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc. Chicago, IL, USA) and Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA). All data are presented as the mean ± standard deviation (SD) of at least three independent experiments. The Mann-Whitney test was used to analyze differences in GNB2L1 immunohistochemistry scores between tumors, CIN, and NC tissues. The relationships between GNB2L1 expression and clinicopathological characteristics were tested by the chi-squared test or Fisher's exact test, as appropriate. GNB2L1 copy number in different tissues was analyzed with a Student's t-test.

Results

GNB2L1 is highly expressed in cervical cancer

GNB2L1 expression levels in 20 cases of CSCC, CIN, and control patients were assessed. GNB2L1 mRNA and protein levels in the CSCC tissues were higher when compared to the CIN and NC tissues (Figure 1A and Figure 1B). To determine if the observed GNB2L1 expression patterns were conserved in CSCC cells, we investigated GNB2L1 expression in SiHa, C33a, and immortalized cervical (H8) cells. The GNB2L1 expression in SiHa and C33a cells was significantly higher than the expression in immortalized H8 cells (Figure 1C). Additionally, when compared to the SiHa cell line, the GNB2L1 protein levels in the C33a cell line were elevated. These results indicated that GNB2L1 is highly expressed in CC patients. To further investigate the clinical significance of GNB2L1 expression in CSCC, we performed immunohistochemistry on CSCC, CIN, and NC tissues. GNB2L1 was significantly upregulated in CSCC tissues when compared to CIN and NC tissues. In addition, GNB2L1 protein expression increased when cervical lesions progressed

from CIN to CSCC (Figure 1D). To further investigate the clinicopathologic significance of GNB2L1 expression in CSCC, the relationship between GNB2L1 expression and its clinicopathologic characteristics was analyzed (Table 1). Upregulation of GNB2L1 expression was significantly and positively related to the clinical stage ($P = 0.017$), lymph node metastasis ($P = 0.049$), and the degree of cell differentiation ($P < 0.001$). No significant correlations between GNB2L1 expression and other clinical parameters, including tumor volume, were observed.

GNB2L1 gene copy number in cervical cancer is higher than in normal cervical tissues

To further understand why increased expression of GNB2L1 occurs in CSCC, copy number data from 40 CC tumors were analyzed and compared to normal adjacent tissues (Figure 2). The GNB2L1 DNA copy number in CC tissues ($297,200 \pm 10,815.061$) was significantly higher than the copy number in NC tissues ($215,100 \pm 7,276.392$, $[T = -2.770, P = 0.009]$).

High GNB2L1 expression is associated with poor cervical cancer prognosis

To investigate the prognostic value of GNB2L1 for CSCC, KaplanMeier analysis and logrank tests were performed. GNB2L1 protein expression and clinical followup data from all 132 patients were used for the analysis (Figure 3). A total of 122 patients died during the followup period, and 10 patients were still alive. The results indicated that the median survival time for patients with high GNB2L1 expression (43.13 ± 1.65 months; $n = 95$) was significantly shorter than the survival time for patients with low GNB2L1 expression (63.39 ± 2.28 months; $n = 37$; $P < 0.001$; Figure 3A). These results revealed that high GNB2L1 expression is indicative of a poor prognosis for CSCC patients. The ROC curve analysis divided the tissue sample scores into “low expression” and “high expression” categories. The cut-off value for high expression tissues was a score >2.34 , and low expression tissues had a cut-off value of ≤ 2.34 .

GNB2L1 signal pathway regulation and gene interaction network

The correlation between GNB2L1 expression level and invasion and metastasis signatures in TCGA, GSE9750 and GSE68339 datasets of cervical cancer was detected by gene enrichment analysis (GSEA). The results showed that the up-regulation of GNB2L1 expression and the signal pathways related to fatty acid metabolism (Akt/mTOR and Jak-STAT) were involved in GNB2L1 (Figure 4A). Indeed, each differential protein is regulated by multiple signaling pathways. Here, we focused on the fatty acid metabolism pathway and selected genes that were important to that pathway. It is already known that the AKT/mTOR and JAK/STAT/PPAR pathways are important for fatty acid metabolism and are associated

with lymph node metastasis. Therefore, we measured lipid accumulation with Oil Red O staining. The staining results indicated that shGNB2L1 cells exhibit decreased lipid levels (Figure 4B).

Discussion

CC remains a major public health problem in developing countries. Importantly, a favorable prognosis and increased long-term survival time are correlated with early CC diagnosis and appropriate therapy [15]. Several studies have demonstrated that abnormal molecular biology can predict the prognosis of tumor patients [16–17]. However, in these studies, no single bio-maker that was used to detect CC was both highly sensitive and specific. In the present study, we focused on GNB2L1 to explore its roles in CC progression and to develop a better biomarker to diagnose and inform the treatment of Uighur women with CC.

We first investigated GNB2L1 expression levels in CSCC, CIN, and NC tissues with qRT-PCR, western blot, and IHC. The results indicated that GNB2L1 expression is dramatically increased in CSCC tissues when compared to CIN and NC tissues. Higher GNB2L1 expression was associated with an advanced FIGO stage, lymph node metastasis, and the degree of cell differentiation. GNB2L1 is reported to be upregulated in multiple cancers including oral squamous carcinoma [19], hepatocellular carcinoma [20], and pancreatic ductal adenocarcinoma [11]. A previous study by Liao and colleagues indicated that overexpression of GNB2L1 promotes CC cell proliferation and invasion by affecting the p53 signaling pathway [21]. However, there are no reports on the prognostic value of GNB2L1 in CC. In the present study, we found that patients with high GNB2L1 expression had shorter overall survival times when compared to patients with low GNB2L1 expression. Thus, our findings indicate that increased GNB2L1 expression predicts a poor CC prognosis.

To elucidate the molecular mechanism of GNB2L1 upregulation in CC cells, we measured changes in GNB2L1 gene copy number with qRT-PCR and sequencing. The results revealed that GNB2L1 DNA copy number in CC tissues was significantly higher when compared to NC tissues.

It is likely that these discrepancies are a result of differential expression of GNB2L1 protein in CSCC.

Although, the GNB2L1 is closely related to the occurrence and development of CSCC, CC tumors are potentially regulated by a different complex multi-gene and multi-molecule network structure. The synergistic effect of various tumor genes promotes the formation and development of CC cells. Therefore, to reveal the molecular mechanisms that drive CC development, the field should focus on individual network pathways that are related to differentially expressed proteins in CC. Changes in the expression patterns of related pathway proteins and the interactions between these proteins should be analyzed. Here, we constructed an interaction network using bioinformatics and the STRING database. These data will provide a starting point for future studies. The analysis identified that the GNB2L1 protein interacts with two pathways in our network map. Based on the bioinformatics study, we propose the AKT/mTOR and JAK/STAT signaling pathways as candidates for further studies.

GNB2L1 has multiple functions. For example, GNB2L1 shuttles proteins and facilitates cross-talk between distinct signaling pathways [22]. Moreover, binding GNB2L1 to specific kinases can result in increased (JNK, MAPKs) or decreased (SRC) catalytic activity [23]. The literature also reports that GNB2L1 has a role in lipid metabolism where it modulates the expression of PI3K and the phosphorylation of AKT [24]. We speculated that GNB2L1 also regulates fatty acid metabolism through the AKT/mTOR signaling pathway. For this reason, we measured lipid accumulation with Oil Red O staining, and our results indicated that lipid levels are reduced in shGNB2L1 cells.

Conclusions

In this study, we demonstrated that GNB2L1 mRNA and protein are higher expression in cervical cancer patients, and it is associated with poor prognosis in cervical cancer patients. Furthermore, copy number data from CC tumors were analyzed and compared to normal adjacent tissues and the results showed that the GNB2L1 DNA copy number in CC tissues was significantly higher than the copy number in NC tissues. From these results, we conclude that the overexpression of GNB2L1 may contribute to tumor progression and it is could be another potential prognostic biomarker for CC patients.

Lists Of Abbreviations

CC: Cervical cancer; GNB2L1: Guanine nucleotide-binding protein subunit beta-2-like 1; CIN:cervical intraepithelial neoplasia

Declarations

Ethics approval and consent to participate

The study protocol was approved by the ethics committee of the First Affiliated Hospital of Xinjiang Medical University (No. 20180226-08) and we collected informed consent forms from all patients.

Consent for publication

Not applicable

Competing interests

The authors have no conflicts of interest to disclose

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Authors' contributions

JQ M and A H are responsible for the study design and preparation of the manuscript. T X recruited the patients and collected samples. LX X, H T and JQ M performed most experiments, analyzed data, and assisted preparation of the manuscript. All authors have read and approved the manuscript.

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Tables

Table 1 Relationship between GNB2L1 protein expression levels in cervical lesion and clinical pathological features of C5CC

Characteristics	n=220	Negative	Weak	Moderate	Strong	χ^2	P
Normal cervical epithelia	32	18	7	6	1	55.832	0.001
CIN I-II	56	19	17	14	6		
CSCC	132	13	28	32	59		
pathological grade							
Well	60	8	21	11	20	15.908	0.001
Moderate /Poor	72	5	7	21	39		
FIGO Stage							
≤II	74	11	20	14	29	10.063	0.017
III A	58	2	8	18	30		
L/N metastasis							
Positive	55	4	6	15	30	7.626	0.049
Negative	77	9	22	17	29		
Tumor diameter							
≤4 cm	79	8	22	19	30	6.176	0.103
>4 cm	53	5	6	13	29		

Figures

Figure 1

GNB2L1 is overexpressed in human CC. (A) Quantitative RT-PCR methods were used to measure GNB2L1 mRNA levels in human CC, CIN, and NC tissues. (B) GNB2L1 protein expression in CSCC, CIN, and NC tissues was analyzed by western blot. (C) GNB2L1 protein expression in C33a, SiHa, and H8 cells was analyzed by western blot. (D) GNB2L1 protein expression was detected with immunohistochemistry in CC, CIN, and NC tissues, as well as the lymph nodes; 1 represents GNB2L1 expression in normal cervical tissues, 2 represents CIN2, 3 represents CIN3, and 4 represents CC, respectively. Data are expressed as the mean $\pm$ standard error of the mean (SEM) for (A), (B), and (C), $**P < 0.01$.

Figure 2

GNB2L1 DNA copy number in CSCC and NC tissues; B represents CSCC tissue and C represents NC tissue. B and C display the sequencing and alignment maps, respectively, ***P < 0.001.

Figure 3

Kaplan Meier curves displaying survival differences between CSCC patients with (A) high GNB2L1 expression (n = 95) and low GNB2L1 expression (n = 37). Survival difference between patients in the high and low GNB2L1 expression groups with (B) early ($\leq$ II) and advanced stage ($>$ II) CSCC were also analyzed. P values were determined using the log rank test. $\chi^2 = 52.984$; P < 0.001; OS, overall survival.

Figure 4

(A) Network analysis of differential CSCC proteins with GNB2L1 as the core. (B) Knockdown of GNB2L1 reduced lipid accumulation.